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In-situ* forming implants loaded with chlorhexidine and ibuprofen for periodontal treatment: proof of concept study *in vivo

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Abstract

Control of infection and inflammation is crucial for the success of periodontal treatment. In this study, *in-situ* forming implants (ISFI) loaded with chlorhexidine dihydrochloride (CHX) and ibuprofen (IBU) were developed and tested to optimize periodontal treatment outcomes. Release profiles were promising. Exposure to 1.5% and 5.3% CHX-IBU loaded ISFI's release media decreased significantly the *P.gingivalis* growth up to 20-fold and 35-fold, respectively, after 48h ($p < 0.05$). The metabolic activity assay of gingival epithelial cells (EC) demonstrated 1.5% CHX-IBU-loaded ISFI to be non-toxic, therefore, it was selected for further experimentation. Furthermore, significant down-regulation of TNF- α release (34% at 6h and 43% at 24h, $p < 0.05$) in *P.gingivalis* lipopolysaccharide (*Pg*-LPS) stimulated EC exposed to 1.5% CHX-IBU ISFI release medium was demonstrated by ELISA. *In vivo*, 1.5% CHX-IBU ISFI was injected into the periodontal pocket in an experimental periodontitis mouse model and the reduction in inflammation and improvement in periodontal wound healing was evaluated through inflammatory cell scoring and histomorphometry at 7- and 15-days post-treatment. The results indicate that CHX-IBU loaded ISFI could be efficient as adjuvant to periodontal therapy for the control of infection and inflammation. Moreover, other (e.g., pro-regenerative) drugs could be incorporated into ISFI to further improve periodontal treatment outcomes.

1. Introduction

Periodontal diseases, comprising gingivitis and periodontitis, are a group of inflammatory diseases of infectious origin that progressively undermine the integrity of the tooth supporting structures leading to tooth mobility and, if left untreated, can result in tooth loss (Kinane et al., 2017). Severe periodontitis has been ranked as the 6th most prevalent disease worldwide affecting around 743 million people and is associated to reduced quality of life (Tonetti et al., 2017). Clinically, such disease manifests itself as inflamed gingiva, bleeding gums, loss of tissue attachment, periodontal pocket formation, bone loss and tooth mobility (Pihlstrom et al., 2005).

Oral biofilms are implicated in the pathogenesis of periodontal diseases (Socransky and Haffajee, 2005). Chronic inflammation triggered by oral pathogens, such as *Porphyromonas gingivalis* (*P.gingivalis*), in periodontal tissues leads to an accumulation of subgingival inflammatory infiltrate and the downwards proliferation of oral epithelial cells forms a junctional epithelium that becomes the epithelial lining of the periodontal pocket (Bosshardt, 2018). Epithelial cells are the first cells to migrate towards the periodontal lesion owing to their high rate of proliferation, hence, their role in the formation of long junctional epithelium and wound healing is crucial and the premature epithelial downgrowth hinders ideal wound healing (Morand et al., 2016; Sculean et al., 2015). The underlying cellular mechanisms implicated in lesion development are regulated by host-immune response and secretion of cytokines that are exacerbated by the infection (Cekici et al., 2014). Therefore, control of inflammation and infection are crucial to the success of periodontal treatment and wound healing.

The conventional periodontal treatment comprises scaling and root planing. It effectively reduces bacterial load and subsequent inflammation; however, its outcomes remain elusive in severe and deep lesions or in the presence of local/systemic risk factors (Tomasi et al., 2007).

Therefore, the use of several anti-inflammatory or anti-infective agents has been proposed as an adjunctive therapy. Regarding inflammation control, ibuprofen (IBU), a selective COX-2 inhibitor, is a well-known anti-inflammatory agent that has been prescribed in periodontal treatment and has shown to improve the treatment outcome upon systemic administration (Agossa et al., 2016). Furthermore, chlorhexidine (CHX) is a widely used broad-spectrum antimicrobial agent that inhibits bacterial growth and is commonly used in patients during periodontal treatment. Several other drugs, compounds or probiotics have also been tested and improved clinical outcomes (Petit et al., 2019; Agossa et al., 2016; Martin-Cabezas et al., 2016; Feres et al., 2015). However, long-term and high dose of systemic drug administration could be associated to side-effects and complications (Petit et al., 2019; Agossa et al., 2016; Greenstein and Polson, 1998). To overcome this issue, the use of local drug delivery systems or scaffolds/carriers has been suggested for controlled and site-specific drug delivery (Batool et al., 2018a; 2018b; Özdoğan et al., 2018). Biodegradable polymers, such as poly(lactic-co-glycolic acid) (PLGA), offer an interesting potential as matrix formers, allowing to control drug release during several weeks (Bragagni et al., 2018; Duque et al., 2018; Wu et al., 2018; Kempe et al., 2010).

In this context, drug-loaded membranes have been tested with promising results, however, their placement requires a surgical approach (Batool et al., 2018a; da Silva et al., 2016; Farooq et al., 2015), paving the way to the use of easy-to-handle materials such as gels or mini-devices/chips with easy intra-pocket administration (Bansal et al., 2018; Nair and Anoop, 2012). Conventional gels can be dislodged from the pocket or lesion site due to salivary flow, chewing or brushing, reducing their efficacy. To overcome such limitation, polylactic-co-glycolic acid (PLGA) and hydroxypropyl methylcellulose (HPMC)-based *in-situ* forming implants (ISFI) have been developed and characterized for local periodontitis application (Agossa et al., 2017, Do et

al., 2014, 2015a, 2015b). The PLGA was dissolved in N-methyl-pyrrolidone (NMP); HPMC and different types of drugs were added. Upon contact with aqueous fluids, the NMP diffuses into the latter and water diffuses into the formulations (NMP and water being completely miscible). Since PLGA is insoluble in water, it precipitates and traps the drug. These liquid formulations possess an appropriate consistency for intra-pocket injection that hardens to become an implant within the pocket, acquiring a complete 3-dimensional filling of the lesion morphology with adequate bio-adhesion and retention at the lesion site. The local administration of these bio-compatible and bio-degradable ISFI is less technique sensitive, less time-consuming, less invasive, consequently, improving patient compliance with convenient drug delivery and optimal at site concentration (Agossa et al., 2017; Do et al., 2014; 2015a; 2015b).

Since periodontitis involves a multifactorial pathogenesis, a treatment targeting both bacterial infection and inflammation could be potentially effective in improving the treatment outcomes. A variety of ISFI loaded with different amounts of IBU (free acid), IBU lysinate, CHX dihydrochloride or CHX digluconate (exhibiting substantially different solubilities) has recently been proposed and the resulting *in vitro* drug release kinetics have been reported (Lizambard et al., submitted). However, it is unclear whether such advanced local drug delivery systems show promising antibacterial and anti-inflammatory activity *in vitro* and *in vivo*. Therefore, the aim of this study was to characterize a selection of IBU-CHX loaded ISFI, evaluating their efficacy to promote periodontal wound healing and regeneration *in vitro* and *in vivo*.

2. Materials & Methods:

2.1. Reagents

Poly(D,L-lactic-co-glycolic acid) (PLGA, 50:50, -COOH end groups; Resomer RG 502 H) and chlorhexidine dihydrochloride (Evonik, Darmstadt, Germany); acetyltributyl citrate (ATBC, Morflex, Greensboro, NC, USA); hydroxypropyl methylcellulose (HPMC, Methocel E50; Colorcon, Dartford, UK); ibuprofen (BASF, Ludwigshafen, Germany). N-methylpyrrolidone (NMP), acetonitrile, sodium phosphate dibasic (Na_2HPO_4) and phosphoric acid (H_3PO_4) (Fisher Scientific, Illkirch, France); triethylamine (for analysis) (Acros Organics, Geel, Belgium). *P.gingivalis* lipopolysaccharide (*Pg*-LPS) (Invivogen, San Diego, CA, USA); brain-heart infusion medium, hemin and menadione (Sigma, St. Louis, MO, USA).

2.2. Preparation of the liquid formulations able to form ISFI

PLGA (28% w/w, based on the total liquid formulation without drug) was dissolved in NMP at 25 °C for 30 min under stirring in a glass vial. ATBC (10% w/w, based on the PLGA mass) and HPMC (10% w/w, based on the PLGA mass) were added and the mixture was vortexed for 3 min followed by standing for 3h at 25 °C. Subsequently, CHX and IBU were added, and the mixtures were vortexed for 3 min. The drug loadings were 1.5% or 5.3% (w/w, based on the *in-situ* implant formulation without NMP = “theoretical” implant composition), as indicated. The formulations were then stored at 2-6 °C and protected from light prior to use.

2.3. ISFI formation, drug release and mass changes *in vitro*

In-situ implant formation: One hundred μL of the liquid formulations were placed at the bottom of an Eppendorf vial using a standard syringe. One mL preheated (37 °C) deionized water

was carefully added into the vial using a pipette, initiating “solvent exchange” and ISFI formation. The vials were horizontally shaken at 37 °C and 80 rpm (GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany).

Drug release: At pre-determined time points, the bulk fluid was completely withdrawn and replaced with fresh deionized water. The drug content in the withdrawn liquid was determined by HPLC-UV (Waters Alliance 2695 separation module, Waters 2489 UV/vis detector) as follows: 20 µL samples were injected into an AC18RP column (Gemini 5 µm C18 110 Å, 100mm x 4.6 mm; Phenomenex, Le Pecq, France). The flow rate was 1.5 mL/min. Eluent A was a 70:30 mixture of an aqueous solution of 115 mM Na₂HPO₄ with 0.5% triethylamine (adjusted to pH 2 with H₃PO₄) and acetonitrile. Eluent B was acetonitrile. The composition of the mobile phase was changed as follows: 4 min Eluent A, 10 min Eluent A:B 55:45. Retention times were approximately 2.50 min for CHX and 6.85 min for IBU. The column was kept at room temperature and the detection wavelength was $\lambda = 239$ nm. The calibration curve was linear ($R > 0.999$) within the range of 1 to 100 µg/mL for CHX and within the range of 1 to 50 µg/mL for IBU. Each experiment was conducted in triplicate, and the results are presented as mean values \pm standard deviation.

2.4. Cell culture

Human oral epithelial cells (TERT-2 OKF-6, BWH Cell Culture and Microscopy Core, Boston, MA, USA) (EC) were cultured in Keratinocyte-SFM medium (Life Technologies, Saint-Aubin, France) as described previously (Batool et al., 2018a).

2.5. Metabolic activity assay

To assess the effect of different doses of CHX-IBU released by ISFI on EC viability, an AlamarBlue assay (Life Technologies, Saint-Aubin, France) was performed as described previously (Batoool et al., 2018a). Prior to the experiment, placebo ISFI (0% CHX-IBU), 1.5% and 5.3% CHX-IBU ISFI were formed as described above for the *in vitro* drug release studies, but using a saliva-like medium described by Pratten et al. (1998) instead of water. After 24h, the bulk fluids were collected and EC were exposed to them during 6 to 24h. Then, 200 μ L of incubation media was transferred to a 96-wells plate and absorbance was measured at OD₅₇₀ and OD₅₉₅ nm by a spectrophotometer (Multiskan, ThermoScientific, Illkirch, France) in order to determine the percentage of AlamarBlue reduction.

2.6. ELISA

To evaluate the anti-inflammatory effects of ISFI, the supernatants from the untreated EC as well as from the cells treated with ISFI placebo and CHX-IBU ISFI were collected at 6 and 24h and subjected to ELISA for the detection of TNF- α concentrations. Prior to the experiments, EC were stimulated by *Pg*-LPS at a concentration of 1 μ g/mL. Recombinant human TNF- α protein (Catalogue number: 300-01A, Peprotech) was used as the standard protein. Anti-Human TNF- α (Polyclonal Goat) (Catalogue number: 500-P31AG, Peprotech) was used as the coating antibody, while biotinylated Anti-Human TNF- α (Polyclonal Goat) (Catalogue number: 500-P31AGBT, Peprotech) was utilized as the capture antibody. ELISA immunoreactivity was quantified using a microplate reader at OD₄₅₀ nm and the standard curve was used to calculate and compare the concentrations of TNF- α protein (pg/mL) in the samples (Figure 1).

2.7. Bacterial culture

The *P.gingivalis* strain (ATCC 33277) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as described previously (Batool et al., 2018a). Briefly, bacterial culture was performed under strict anaerobic conditions at 37°C in brain-heart infusion medium supplemented with hemin (5 mg/mL) and menadione (1 mg/mL). For each experiment, bacteria were grown for 4 days. Before use, the bacterial culture was centrifuged and bacteria were washed twice with phosphate buffer saline (PBS).

2.8. Effect on bacterial growth over time

Prior to evaluating the effect of the ISFI on *P.gingivalis* growth, bacterial growth was evaluated at OD₆₀₀ nm using a spectrophotometer. *P.gingivalis* growth was recorded in bacterial culture medium (control), placebo ISFI, CHX-IBU ISFI release media respectively and compared to determine the effect of CHX-IBU ISFI as described previously (Akcali et al., 2014). Growth rate was evaluated at 6, 9, 24 and 48h (Figure 1).

2.9. Experimental periodontitis

Twenty-four 8 weeks old male C57BL/6J mice (mean weight= 25g) (Charles River, L'Arbresle, France) were used in this study. All animals were regularly fed and kept in separate cages. All experimental protocols fulfilled the authorization of the "Ministère de l'Enseignement Supérieur et de la Recherche" under the agreement number 01715.02. The Ethics Committee of Strasbourg named "Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg (CREMEAS)" specifically approved this study. Mice were examined to evaluate pain and stress and their weights were monitored daily. Experimental periodontitis was induced in mice by *P.gingivalis*-infected ligatures to mimic disease condition comparable to human periodontitis as described previously (Saadi-Thiers et al., 2012). Briefly, silk ligatures (6-0) were

incubated in *P.gingivalis* culture at 37 °C overnight. After anaesthesia, *P.gingivalis* infected ligatures were placed repeatedly in the palatal sulcus of the first molars bilaterally. The initial placement of *P.gingivalis*-infected ligatures was facilitated by sulcular incisions along the cervical margins of the first molars. The ligatures were replaced with freshly infected ones thrice a week for up to 40 days to create deep periodontal defects (Figure 1).

2.10. Treatment of periodontal defects

Periodontal defects were randomly treated with intra-pocket administration of ISFI (placebo or 1.5% CHX-IBU) or left untreated (natural wound healing/ control). The consistency of ISFI was liquid enough for easy injectability which later solidified to mould itself according to the lesion's shape and size and deliver the therapeutically active molecules. A 21-gauge needle was used to inject the liquid formulations into the periodontal pockets. Prior to injection, the lesion area was well-isolated using tiny pellets of cotton to absorb saliva or blood. **To ensure ISFI fitting to the 3D shape of the lesion, 100 µl of ISFI were injected slowly in each lesion and excess were gently removed. Mouse's mouth was maintained open 5 minutes after injection and ISFI placement was checked under microscope.** The margins of the pockets facing the cervical margins of the teeth were then closed to allow the ISFI formation. Mice were euthanized at 7 days (n=12) and 15 days (n=12) post-treatment (Figure 1).

2.11. Tissue preparation

Mice were euthanized with an intraperitoneal lethal injection of pentobarbital (100mg/kg) (Centravet). Tissue fixation was performed by intra-cardiac perfusion with a solution containing 4% paraformaldehyde (PFA) in PBS (pH 7.4). Afterwards, maxillae were dissected

and post-fixed by immersion in the same fixative solution overnight at 4°C. After rinsing with PBS for 24h, the specimen were demineralized at 4 °C in 15% EDTA at pH 7.4 for 3 weeks with a regular replacement of solution every 2 days. After extensive washing in PBS, the samples were dehydrated in increasing concentrations of ethanol and toluene before embedding in paraffin (Paraplast plus, Sigma, city, country). Seven µm thick serial frontal paraffin sections of the maxilla were cut with a microtome (Leica RM 2165).

2.12. Inflammatory score

The 7 µm thick paraffin slides were deparaffinized in xylene and rehydrated through a series of graded ethanol, stained using standard methods with hematoxylin and eosin, and then evaluated for inflammatory changes. The semiquantitative grade of inflammation in gingival tissues (Shi et al., 2015) was used to give an inflammatory score to each section. The scale ranged from 0 to 3: 0 = no inflammation, no inflammatory cell infiltration; 1 = mild inflammation, local focal inflammatory cell infiltration; 2 = moderate inflammation, spotty and flaky inflammatory cell infiltration; and 3 = severe inflammation, diffuse inflammatory cell infiltration. The grade of inflammation of 3 specimens per mouse per condition was measured and the average grade of inflammation was calculated. The sections were visualized and photographed with a Leica DM2500B microscope (Leica Microsystems, Wetzlar, Germany).

2.13. Histomorphometric analysis

For histomorphometric evaluation, prepared sections were deparaffinized, rehydrated, and stained with hematoxylin. After dehydration, slides were mounted with distrene–plasticizer–xylene (DPX) resin (Sigma, city, country) and computerized images were captured on a microscope (RM 2145 DMRB microscope, Leica, Rueil-Malmaison, France). Palatal root areas of

the first molars were analysed to examine the extension of epithelial down growth, fibrous connective tissue attachment and alveolar bone loss. For histomorphometric analysis: epithelial attachment (EA) was measured from the cemento-enamel junction (CEJ) to the apical limit of the epithelium; fibrous connective tissue attachment (FA) was measured from the base of the apical limit of EA up till the beginning of the dense fibrous connective tissue fibers, and the alveolar bone loss (BL) was measured from CEJ to alveolar bone crest (ABC) using the Image J imaging software (Schneider et al., 2012).

2.14. Statistical analysis

All experiments were repeated at least three times in triplicates (technical and biological replicates), and statistical analysis was performed using ANOVA and Mann-Whitney U test. A probability of a p -value < 0.05 was considered significant.

3. Results

3.1. Release kinetics of IBU and CHX from ISFI

As it can be seen in Figure 2, the investigated ISFI released both drugs (IBU and CHX) in a controlled manner. IBU release was about constant: approximately 9 and 28 µg/day in the case of 1.5% and 5.3 % ISFI. The release rate of CHX was higher during the first 5 days, and then slowed down.

3.2. ISFI reduced *P.gingivalis* growth in vitro

To evaluate the antibacterial effect of the investigated ISFI, 5.10^7 CFU in 100 µL of *P.gingivalis* culture was incubated with 100 µL samples of release media obtained with placebo, 1.5% and 5.3% CHX-IBU ISFI. The samples were obtained after 1 day of exposure of the ISFI to artificial saliva. After 9 to 48h incubation, *P.gingivalis* growth over time was significantly reduced in comparison with unaltered bacterial culture (Figure 3). Treatment with 1.5 and 5.3% CHX-IBU ISFI's release media reduced the bacterial growth by about 2-fold and 4-fold at 6 and 9 hours respectively ($p<0.05$). An 18-fold decrease in the *P.gingivalis* growth was observed at 24h while it further decreased to 20-fold at 48h with exposure to 1.5% CHX-IBU ISFI release medium ($p<0.05$). Similarly, 5.3% CHX-IBU ISFI's release medium also induced a significant decrease of *P.gingivalis* growth by upto 24 and 35-fold at 24h and 48h respectively. Interestingly, the release medium of placebo ISFI also induced a moderate antibacterial effect which can be attributed to the antibacterial effect of PLGA or NMP.

3.3. Anti-inflammatory effect of ISFI's release

The effect of CHX-IBU ISFI on the metabolic activity of EC was determined to evaluate their cytocompatibility. EC were incubated for 6 and 24h with samples of release media obtained

with placebo ISFI, 1.5% and 5.3% CHX-IBU ISFI after 1 day of exposure to artificial saliva. It should be noticed that EC metabolic activity was not significantly modified in the case of 1.5% CHX-IBU ISFI at both 6 and 24h, while a slight decrease in the metabolic activity was observed for EC exposed to release medium obtained with 5.3% CHX-IBU ISFI (Figure 4A). Hence, 1.5% CHX-IBU ISFI was selected for further experimentation owing to its non-cytotoxicity.

To evaluate the anti-inflammatory effects of the ISFI, EC were first exposed to *Pg*-LPS to induce an inflammatory state and then incubated for 6 to 24h with samples of release media obtained with ISFI after 1 day of exposure to artificial saliva. *Pg*-LPS stimulation induced a significant TNF- α secretion at 6 and 24h (Figure 4B, C). At 6h, exposure to the release medium obtained with 1.5% CHX-IBU ISFI reduced TNF- α release from *Pg*-LPS stimulated EC by 34% in comparison to that of the control, while a 43% reduction was observed at 24h ($p < 0.05$). Such decreases were not observed for EC treated with placebo ISFI, illustrating the role of released IBU (Figure 4).

3.4. Intra-pocket application of ISFI reduced inflammation associated to experimental periodontitis

Due to its promising bactericidal and anti-inflammatory properties *in vitro*, 1.5% CHX-IBU ISFI was evaluated *in vivo* in a murine experimental periodontitis model as a proof-of-concept. Following establishment of periodontal lesions, 1.5% CHX-IBU ISFI was injected atraumatically into the periodontal pocket. During the procedures and follow-up, neither significant weight changes nor any adverse events were observed. At 7 days post-ISFI application, within epithelial and connective tissue compartments of the gingiva, a significant reduction in inflammatory score was observed in sites treated with 1.5% CHX-IBU ISFI in

comparison with untreated sites and sites treated with placebo ISFI (59% vs untreated and 56% vs placebo, $p < 0.05$) (Figures 5). A significant reduction in inflammatory score of 55% and 53% was also observed 15 days post-ISFI treatment in sites treated with 1.5% CHX-IBU ISFI in comparison with untreated sites and in sites treated with placebo ISFI respectively (Figures 6). Additionally, histomorphometric analyses showed improvement in periodontal wound healing at 7 days (Figure 7) and 15 days (Figure 8), as exhibited by significantly shorter EA in sites treated with CHX-IBU ISFI (41% vs untreated and 22% vs placebo, $p < 0.05$) at 7 days (Figures 7D) and at 15 days (50% vs untreated and 47% vs placebo, $p < 0.05$) (Figure 8D). Furthermore, at 7 days, the FA on the root surface was significantly improved by 24% and 45% in 1.5% CHX-IBU ISFI treated sites in comparison to the placebo ISFI and untreated controls respectively ($p < 0.05$) (Figures 7E) and at 15 days (28% vs placebo and 52% vs untreated, $p < 0.05$) (Figure 8E). The connective tissue fibers appeared to be denser and well-organized along the root in the sites treated with 1.5% CHX-IBU ISFI. No significant differences were observed in terms of bone healing at both time points studied (Figures 7F and 8F).

4. Discussion

Control of periodontal inflammation is the main goal of periodontal therapy and is achieved after oral biofilm removal. However, this aim could be difficult to achieve due to the presence of modifiable and non-modifiable risk factors and in some cases, exacerbated inflammation persists at lesion site and induces significant tissue destruction. Therefore, the development of easy-to-handle pharmacological strategies with low risk of side effects is required as adjunctive treatment. Here, we demonstrated, as a proof-of-concept, the potential use of an ISFI to deliver efficiently active drugs at lesion site *in vivo*.

To allow sustained release of active drugs in periodontal lesions, several types of delivery systems have been developed such as gels (Işıl Özdöğün et al., 2018), fibers, chips (Lecic et al., 2016), membranes (Batool et al., 2018a) and ISFI (Agossa et al., 2017; Do et al., 2014; Kilicarslan et al., 2014). Such advanced drug delivery systems have been loaded with antimicrobials such as antibiotics or antiseptics (Do et al., 2014; Kilicarslan et al., 2014; Lecic et al., 2016), anti-inflammatory compounds (Batool et al., 2018a; Christian et al., 2019; Yar et al., 2016) and pro-regenerative factors such as growth factors (Li et al., 2017). In this study, ISFI loaded with both anti-inflammatory and antimicrobial drugs were evaluated. The investigated ISFI displays major features appropriate for periodontal application. They are biodegradable, injectable and fit the tridimensional shape of the patient's periodontal lesion. With certain, injectable PLGA-based delivery systems important initial burst effects have been reported, followed by sustained release over hours to even weeks (Hatefi and Amsden, 2002). Generally, the incorporated drugs are to be released in a time-controlled manner to provide sustained pharmacodynamic effects over prolonged periods of time (Do et al., 2015a; Kilicarslan et al., 2014).

PLGA is a well-known and well-characterized biodegradable and biocompatible matrix forming material capable to control drug release. However, the mechanical properties of PLGA-based drug delivery systems are generally poorly adapted to the dynamic environment of gingival tissue (Do et al., 2015b; Fisher et al., 2016; Gasmi et al., 2016; Gentile et al., 2014). Therefore, plasticizers and fillers are added to improve the mechanical properties and bio-adhesion. HPMC is a hydrophilic polymer that is added to the ISFI to improve their bio-adhesion, as demonstrated previously (Agossa et al., 2017; Do et al., 2015b).

The possibility to provide good syringeability, similar to water, moderate hardness of the resulting ISFI, good mechanical strength associated to strong adhesion to tooth surface make ISFI a more convenient and efficient mode of intra-pocket drug delivery. The liquid nature of ISFI at the time of injection and a moderately hardened consistency acquiring the 3D geometry of the periodontal pocket later are crucial for the success of the treatment as this process of implant hardening ensures reliable and long-term retention of ISFI along with the drugs.

In this proof-of-concept study, the anti-inflammatory effects of ISFI loaded with IBU and CHX were evaluated. Recently, ISFI have been widely tested for many different applications. For instance, anti-inflammatory (lornoxicam) loaded PLGA based ISFI have been studied in a rat model to treat osteoarthritis related inflammation (Christian et al., 2019). Likewise, controlled release of dexamethasone from ISFI has also been tested for intra-ocular delivery (Bode et al., 2018). As mentioned previously, reduction of inflammation orchestrated by decreased pro-inflammatory cytokine secretion is the aim of periodontal treatment (Cekici et al., 2014). Herein, we demonstrated that the dose of IBU released from the investigated ISFI under the given conditions reduced significantly TNF- α secretion induced by *Pg*-LPS (34% at 6h and 43% at 24h, $p < 0.05$) in EC without any significant cytotoxicity. EC are key cells in periodontal

pathogenesis, as they are considered the first line of defense continuously challenged by periodontal pathogens (Amano, 2007), while *Pg*-LPS is a well described virulence factor that triggers the release of pro-inflammatory mediators in oral and immune cells (Bian et al., 2016; Kim et al., 2018; Morand et al., 2015). This *in vitro* anti-inflammatory effect was corroborated *in vivo*, as a reduced inflammatory score was measured at lesion-sites treated with IBU-CHX loaded ISFI (59% and 55% vs untreated controls at 7 and 15 days respectively, $p < 0.05$). As persistence of soft tissue inflammation is implicated in underlying alveolar bone destruction and impaired wound healing, an improved bone healing was expected. However, such observation was not made and constitutes a limitation of this study. Same trend of result was observed in another study with another type of IBU loaded drug delivery system (Batoool et al., 2018a). Therefore, *in vivo* kinetics of ISFI resorption and drug release should be considered for future studies with a longer follow-up and with a specific insight on bone remodeling and healing.

Long-term use of antibiotics at their minimum inhibitory concentration's (MIC) threshold causes bacterial resistance against several drugs (da Silva et al., 2016; Paolantonio et al., 2009). For instance, the antibacterial spectrum of an antibiotic (doxycycline) loaded injectable formulation based on PLGA was increased by the addition of an antiseptic (secnidazole), however, the resistance of anaerobic bacteria towards doxycycline still posed a problem (Gad et al., 2008). To overcome these limitations, antimicrobial agents such as CHX offer an alternative as adjunct to periodontal therapy. Interestingly, subgingival administration of metronidazole gel and CHX gel has also been studied and showed comparable effectiveness in reducing clinical bacterial load and periodontal pocket depth (Perinetti et al., 2004). Importantly, in our study, effective results have been achieved with lower CHX loadings as compared to other CHX based delivery systems for periodontal treatment (Agossa et al., 2017; Heasman et al., 2001; John et al., 2015; Perinetti et al., 2004).

In this study the effect of CHX-IBU ISFI on periodontal inflammation has been demonstrated both *in vitro* and *in vivo*. To further corroborate the efficacy of CHX-IBU ISFI, it would be interesting to explore its impact on the quantity and quality of oral biofilm *in vitro* and *in vivo*, especially in comparison to the commercially available products or other antibiotic loaded gels. In this study, well-described drugs CHX and IBU were selected as a proof of concept model, nevertheless, incorporation of growth factors and other pro-regenerative molecules could also be tested.

5. Conclusion

The investigated IBU-CHX loaded ISFI offer a promising potential for the local delivery of adjunctive drugs in the treatment of periodontal lesions and open perspectives to develop ISFI loaded with specific molecules, drugs or growth factors that can be combined to enhance bone regenerative response as well to optimize a coordinated healing response of the periodontal soft and hard tissues.

6. Acknowledgments

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7. Conflicts of interest

The Editor-in-Chief of the journal is one of the co-authors of this article. The manuscript has been subject to all of the journal's usual procedures, including peer review, which has been handled independently of the Editor-in-Chief.

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Figure legends

Figure 1: Experimental design

Figure 2: In vitro drug release from the investigated ISFI: (A) IBU release from 1.5% and 5.3% CHX-IBU ISFI; (B) CHX release from 1.5% and 5.3% CHX-IBU ISFI. Results are reprinted (adapted) from Lizambard et al., submitted.

Figure 3: Effect of CHX-IBU ISFI on *P.gingivalis* growth over time. *P.gingivalis* were incubated for 6h, 9h, 24h and 48h with samples of the release media obtained with placebo ISFI, 1.5% and 5.3% CHX-IBU ISFI after 1 day of exposure to artificial saliva. The fold changes in the numbers of *P.gingivalis* are shown. Data are expressed as the mean \pm SD; * $p < 0.05$.

Figure 4: Effect of CHX-IBU ISFI on inflammation and metabolic activity of EC. (A) Metabolic activity of CHX-IBU ISFI treated EC at 6 and 24 hours; (B) TNF- α release from CHX-IBU ISFI treated EC at 6h; (C) TNF- α release from CHX-IBU ISFI treated EC at 24 h; data are expressed as the mean \pm SD. † Difference between non-stimulated and stimulated cells, $p < 0.05$; * difference between 1.5% CHX-IBU ISFI treated cells with or without Pg-LPS stimulation, $p < 0.05$.

Figure 5: Effect of CHX-IBU ISFI on inflammation in a periodontitis-induced mouse model at 7 days of periodontal wound healing. Inflammatory cell count in histological sections of (A) untreated (B) placebo ISFI (C) 1.5% CHX-IBU ISFI. (D) **Inflammatory score:** the scale ranged from 0 to 3: 0 = no inflammation, no inflammatory cell infiltration; 1 = mild inflammation, local

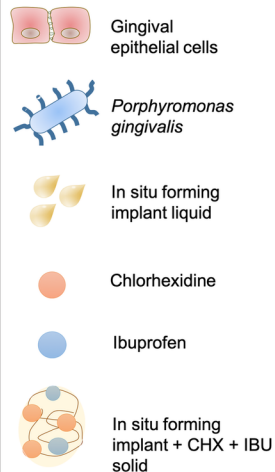
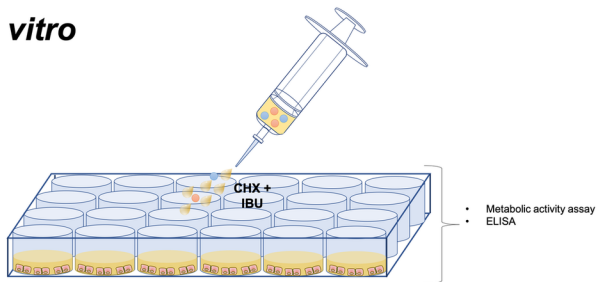
*focal inflammatory cell infiltration; 2 = moderate inflammation, spotty and flaky inflammatory cell infiltration; and 3 = severe inflammation, diffuse inflammatory cell infiltration. Data are expressed as the mean \pm SD. * decrease in inflammatory score by treatment with 1.5% CHX-IBU ISFI, $p < 0.05$.*

Figure 6: Effect of CHX-IBU ISFI on inflammation in a periodontitis-induced mouse model at 15 days of periodontal wound healing. *Inflammatory cell count in histological sections of (A) untreated (B) placebo ISFI (C) 1.5% CHX-IBU ISFI. (D) Inflammatory score: the scale ranged from 0 to 3: 0 = no inflammation, no inflammatory cell infiltration; 1 = mild inflammation, local focal inflammatory cell infiltration; 2 = moderate inflammation, spotty and flaky inflammatory cell infiltration; and 3 = severe inflammation, diffuse inflammatory cell infiltration. Data are expressed as the mean \pm SD. * decrease in inflammatory score by treatment with 1.5% CHX-IBU ISFI, $p < 0.05$.*

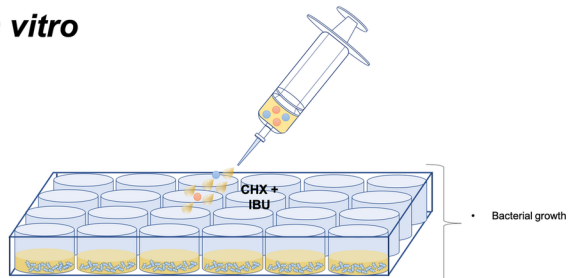
Figure 7: Effect of CHX-IBU ISFI on periodontal wound healing at 7 days in a periodontitis-induced mouse model. *Histological section of (A) untreated; (B) placebo ISFI; (C) 1.5% CHX-IBU ISFI. Red lines = cemento-enamel junction (CEJ); green lines = epithelial attachment level (EA); yellow lines = fibrous connective tissue attachment level (FA); blue lines = bone loss (BL); EPI: gingival epithelium, CT: gingival connective tissue, AB: alveolar bone, R: root, EA: epithelial. Histomorphometric analysis of (D) epithelial attachment level (EA); (E) fibrous connective tissue attachment level (FA); (F) bone loss (BL). Distances are expressed as the mean \pm SD in μm ; * $p < 0.05$.*

Figure 8: Effect of CHX-IBU ISFI on periodontal wound healing at 15 days in a periodontitis-induced mouse model. Histological section of (A) untreated; (B) placebo ISFI; (C) 1.5% CHX-IBU ISFI. Red lines = cemento-enamel junction (CEJ); green lines = epithelial attachment level (EA); yellow lines = fibrous connective tissue attachment level (FA); blue lines = bone loss (BL); EPI: gingival epithelium, CT: gingival connective tissue, AB: alveolar bone, R: root, EA: epithelial attachment. Histomorphometric analysis of (D) epithelial attachment level (EA); (E) fibrous connective tissue attachment level (FA); (F) bone loss (BL). Distances are expressed as the mean \pm SD in μ m; * $p < 0.05$.

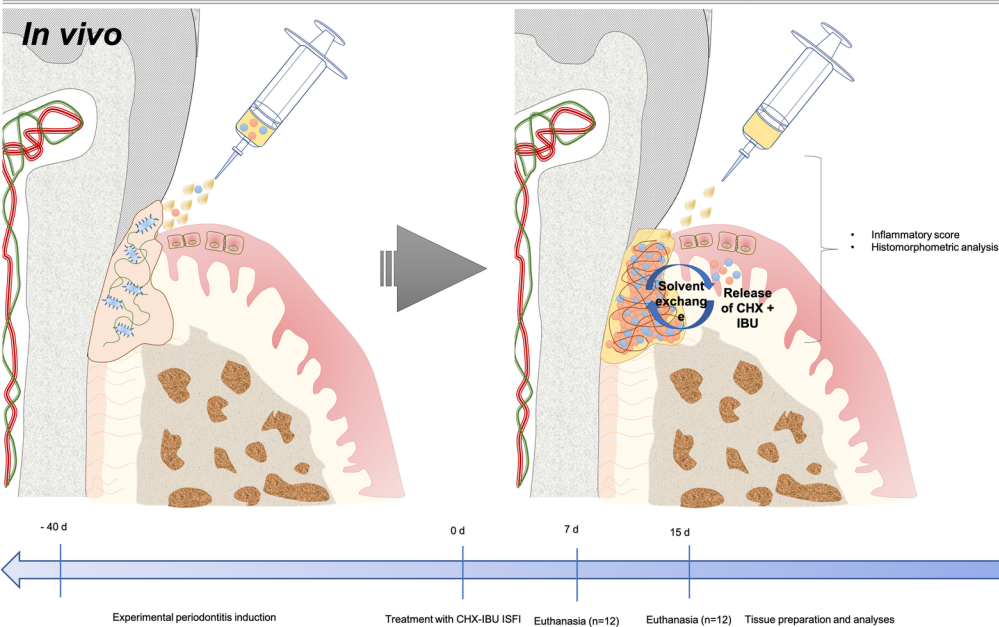
In vitro

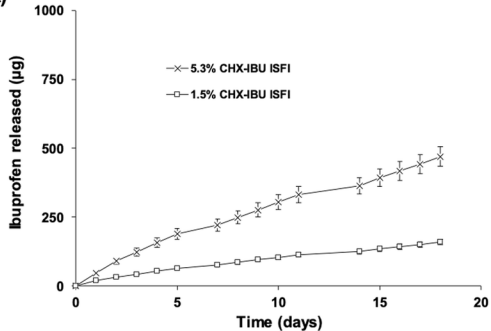
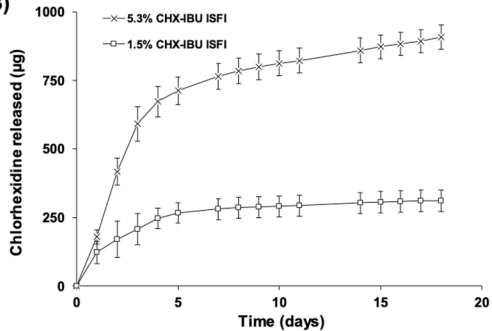


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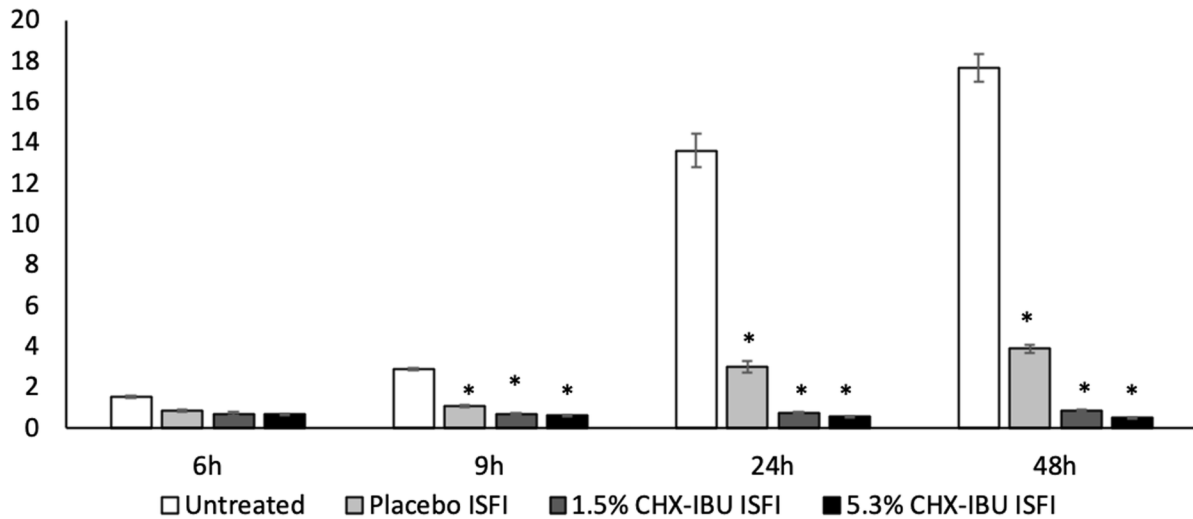


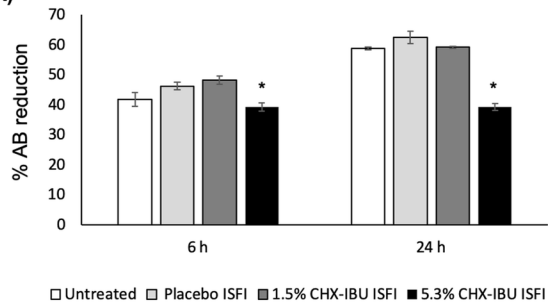
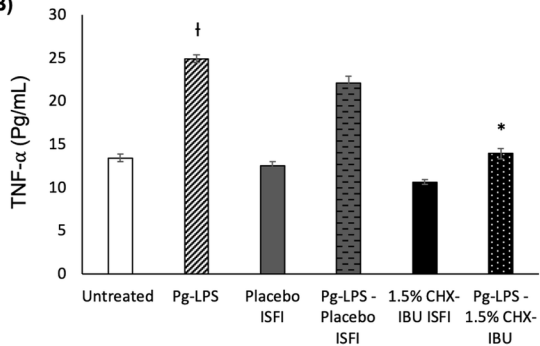
In vivo



A)**B)**

Fold change



A)**B)****C)**